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Influence of a temporary restriction of dietary protein in prepubertal ewe lambs on first lactation milk traits and response to a mammary gland inflammatory challenge

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ABSTRACT

This study evaluated the influence of a temporary nutritional protein restriction (NPR) performed, under commercial conditions, in prepubertal female lambs on first lactation milk production traits and the inflammatory response triggered by an inflammatory challenge of the. From 40 Assaf female lambs, we defined a control group (**C** $n = 20$), which received a standard diet for replacement lambs and the **NPR** group (n = 20), which received the same diet but without soybean meal between 3 and 5 months of age. About 150 days after lambing, 24 of these ewes (13 NPR, 11C) were subjected to an intramammary infusion of *E. coli* lipopolysaccharide (**LPS**). Our dynamic study identified indicator traits of local (SCC) and systemic (rectal T^a, IL-6, CXCL8, IL-10, IL-36RA, VEGF-A) response to the LPS challenge. The NPR did not show significant effects on milk production traits and did not affect the SCC and rectal T^a after the LPS challenge. However, the NPR had a significant influence on 8 of the 14 plasma biomarkers analysed, in all the cases with higher relative values in the C group. The effects observed on VEGF-A (involved in vasculogenesis during mammary gland development and vascular permeability) and IL-10 (a regulatory cytokine classically known by its anti-inflammatory action) are the most remarkable to explain the differences found between groups. Whereas further studies should be undertaken to confirm these results, our findings are of interest considering the current concern about the future world's demand for protein and the need for animal production systems to evolve toward sustainability.

1. Introduction

The world's population continues to grow. From an estimated eight billion people worldwide in 2023, the medium-variant projection indicates that the global population could grow to approximately 8.5 billion in 2030, 9.7 billion in 2050, and 10.9 billion in 2100 (Nations, U, 2019). This, together with other changes such as worldwide increased incomes and urbanisation, will determine changes in the amounts of food needed, the types of foods demanded and their relative contributions to diets (Henchion et al., 2017). Projected demand for protein is of particular interest, with estimations suggesting that the world demand for animal-derived protein will double by 2050 (Westhoek et al., 2011). This trend has led to concerns for sustainability and food security (Henchion et al., 2017), especially in light of increasing global protein prices.

Nutrition is one of the most critical environmental factors affecting phenotypes of interest in livestock, such as production potential, product quality and health traits (Mackle et al., 1999). Thus, obtaining sufficient feed for animals and reducing competition between humans and livestock species for high-quality protein ingredients, such as soybean meal, are growing sustainability challenges for the food production system (Verbeke et al., 2015). In addition, nitrogen emissions from livestock are considered a serious environmental problem, as they are related to hotspots of air and water pollution (European Commission, 2013). Because a large proportion of the total nitrogen consumed by livestock is lost through excretion, reducing dietary crude protein could

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help to reduce nitrogen excretion and, consequently, the environmental impact of current animal production systems.

Dairy livestock species have been used for centuries to provide human milk and dairy products. A fundamental consideration is that the milk yield potential of dairy animals is determined during the pubertal phase, when the growth of the mammary gland (previously isometric) becomes allometric (Sinha and Tucker, 1969). Concerning this critical period, a study showed that heifers raised on high planes of nutrition (i.e., ad libitum feeding) during the allometric phase of mammary development had less secretory tissue in their mammary glands than those raised at 60% ad libitum feeding. In contrast, rapid growth after the allometric period did not affect mammary secretory tissue (Sejrsen et al., 1982). Similar results were reported in dairy sheep (Johnsson and Hart, 1985). In this species, Villeneuve et al. (2010) also reported that restricted feeding before puberty improved mammary gland development without compromising growth performance in ewe lambs. Regarding studies specifically assessing the influence of a protein-restricted diet at the prepuberal age on milk production, Zhang et al. (1995) evaluated, in prepubertal ewes, the effects of two dietary protein levels (15% vs. 20%, $n = 10$ vs. 10) from weaning to puberty on mammary growth, hormone secretions, and milk yield during the first lactation. These authors reported a nonsignificant trend for increased milk yield for ewes fed the 20% protein diet compared with those ewes fed the 15% protein diet when they were prepubertal lambs.

In addition, the worldwide impact of mastitis on dairy cattle and dairy sheep production systems is well known due to the high frequency and related costs of this disease (Barkema et al., 2009; Bergonier et al., 2003). In the pathogenesis of mastitis, a critical role is played by the innate immune response, which is the first line of defence following the invasion of the mammary gland. After this invasion, macrophages accumulate at the infection site, releasing pro- and anti-inflammatory mediators such as cytokines and other molecules that may act locally and systemically and produce local or systemic inflammatory responses (Turk et al., 2017). Cytokines are immunomodulatory polypeptides that participate in adaptive and innate immune responses. Research involving cytokines is essential to understanding the immune system and its multifaceted responses to antigens, especially those that trigger inflammation. In sheep, the well-known relationship between metabolism and mastitis resistance has been proven by Bouvier-Muller et al. (2016), who showed that animals divergently selected for resistance or susceptibility to mastitis had a different response to postpartum negative energy balance. Considering the importance of the allometric development of the mammary gland at puberty, it would also be interesting to determine whether different feeding levels or restriction diets during that period may increase the susceptibility of the udder to clinical or subclinical mastitis.

Mastitis is often associated with reduced milk yield and the occasional involuntary culling of affected animals, resulting in substantial losses in dairy production. In lactating sheep, the literature suggests a link between nutrition and mastitis (Pulina and Bencini, 2004); the significant impact of nutrition on udder health occurs via suppression of immune responses (O'Rourke, 2009). Taking all these points into account, the objective of this work was to evaluate whether a temporary dietary protein restriction performed in prepubertal female lambs under the conditions of a commercial flock, could influence first lactation dairy traits of interest and also the response against an inflammatory challenge of the mammary gland. For this purpose, we performed two experimental challenges. First, a temporary nutritional challenge performed in ewe lambs coinciding with the allometric growth period of the mammary gland (from 3 to 5 months of age), and second, an intramammary gland inflammatory challenge performed at the end of the ewes' first lactation. We believe that the results reported here may be of interest in view of the current concern about the future world demand for protein, and, in turn, the need for animal production systems to evolve toward sustainability.

2. Materials and methods

2.1. Ethics approval

The nutritional challenge described in this work was approved by the Ethics Committee of the *Instituto de Ganadería de Montaña* (**IGM**, Reference 100,102/2018–1) and the corresponding department of the *Junta de Castilla y León* regional government (Resolution 03/08/2018, Agriculture and Livestock Department, *Junta de Castilla y León*, Spain). Likewise, the inflammatory challenge was approved by the Animal Experimentation and Welfare Subcommittee of the University of León (Reference OEBA-ULE-013-2019) and the corresponding department of the *Junta de Castilla y León* regional government (Resolution 23/01/2020, Agriculture and Livestock Department, *Junta de Castilla y León*, Spain).

2.1.1. Nutritional challenge design and milk production phenotypes

We acquired 40 Assaf female lambs (2 months of age) from one flock located in the northwest region of *Castilla y León* (Spain). The animals were transported to the facilities of the IGM in León (Spain). All animals were fed a standard diet for replacement ewe lambs providing 16% crude protein until they were 3 months old. Then, they were divided into two groups of similar average body weight (**BW**): Control (C, $n = 20$; average BW = 29.1 kg) and Nutritional Protein Restriction (NPR, n = 20; average BW = 28.3 kg) groups. Hence, managing the animals in two groups, for 64 days, the C ewe lambs received the standard diet, whereas the NPR ewe lambs received the same diet but without soybean meal, which meant a 44% reduction in protein intake (details on the ingredients and chemical composition of the diets provided during the NPR experiment are given in Table 1). The diet of the NPR group was designed to mimic a feed restriction challenge taking place in a commercial flock due to a trade market problem and a shortage of concentrate inputs, and so, both groups had access to barley straw ad libitum. After this challenge, all the animals were managed in a single group and fed with the same diet until the end of the study according to standard commercial practices based on the animals' needs across the different physiological stages. The ewes were artificially inseminated at 10 months of age. Animals were subjected to standard periodic veterinary treatments (e.g., vaccines and anthelmintic treatments), and their health status was routinely monitored throughout the study. Ewe lambs were also weighed periodically across the experiment, with a total of 8

Table 1
Ingredients and chemical composition of the diet provided to the two groups under study during the temporary nutritional challenge (age 3 to 5 months): control group (C), and nutritional protein restriction group (NPR).

	Control feed	NPR challenge feed (without soybean meal)
Ingredients (g/kg FM[1])		
Maize grain	400	503
Barley grain	300	377
Soybean meal 47	180	–
Wheat bran	60	60
Lard	10	10
Molasses (beet)	20	20
Minerals and vitamins	30	30
Total	1000	1000
Chemical composition (g/kg DM[2])		
Dry matter (g/kg FM)	869	870
Ash	61.0	51.8
Crude protein	182	105
Neutral detergent fibre	119	121
Acid detergent fibre	46.5	39.1
Acid detergent lignin	6.6	8.5
Ether extract	33.8	43.8
Metabolizable energy (Mcal/kg DM)	3.06	3.02

[1] FM: Fresh matter; [2]DM: dry matter.

measurements covering the different stages of the study. Information about the average BWs of the 40 animals across the most important stages of this study, differentiated by diet group (C and NPR) is provided in Supplementary Material S1 (Table S1). A graphical representation of the average BW evolution across the experiment is presented in Supplementary Material S1 (Fig. S1). The difference in BW between the two groups was only significant at the end of the nutritional challenge (see Supplementary Material S1, Table S1). After lambing, milk production was recorded daily, whereas milk samples for milk composition analysis were regularly collected for each ewe from weeks 1 to 12 after parturition (approximately 90 days). Hence, 27 records for each animal, including information on milk yield (**MY**) and milk protein and fat percentages (**PP** and **FP,** respectively), were available for later study. Finally, estimates of MY, PP and FP adjusted to 90 days of lactation (referred to as $\mathbf{MY_{adj90d}}$, $\mathbf{PP_{adj90d}}$, $\mathbf{FP_{adj90d}}$) for each ewe were obtained from the available test-day records using the Fleischman method (Barillet, 1985), and the basic statistics for these three adjusted traits (mean and standard deviation) are shown in Supplementary Table S2.

2.1.2. Intramammary inflammatory challenge and sampling protocol

At approximately day 150 of their first lactation (approximately 20 months of age), 24 ewes from the NPR experiment (13 from the NPR group and 11 from the C group) were selected to be challenged with an intramammary gland infusion of *Escherichia coli* LPS. Twenty-four hours before the LPS challenge, the somatic cell count (**SCC**) (x10^3 cells/mL) was measured for milk samples obtained from each half-udder of the 24 ewes. The half-udder showing the lowest SCC value was the one selected to be challenged with the LPS infusion (note: the SCC averages at the −24 h sampling point were 119 × 10^3 cells/mL for the LPS half-udders and 193 × 10^3 cells/mL for the contralateral half-udders). No significant differences were detected between the two groups of half-udders when the corresponding SCC values (log_{10}-transformed data) were analysed with a *t*-test ($P < 0.09$). A sterile solution of LPS (10 μg/mL, ultrapure LPS, Invivogen, Toulouse, France) was prepared in PBS (Life Technologies SAS, Saint Aubin, France) containing 0.1% bovine serum albumin (Sigma–Aldrich, Saint-Quentin Fallavier, France). Before the administration of the solution, both glands were manually emptied of milk, and the selected teat was disinfected with a compress impregnated with 70% ethanol. The teat canal of the treatment gland was catheterised before injecting 1 mL of the LPS solution. Immediately after injection, the cannula was removed, and the udder was massaged to facilitate diffusion of the solution. From the 24 animals subjected to the experimental inflammatory challenge, the following measurements and samples were collected at various times related to the LPS injection:

1. Measurement of rectal temperature ($\mathbf{T^a}$): The rectal temperature was recorded one day before the LPS injection (−24 h), just before the injection (0 h), and at different time points after the LPS injection (2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 72 h, and 144 h).
2. Plasma samples and analysis with a cytokine/chemokine multiplex immunoassay kit panel: venous blood was collected by jugular venipuncture from the 24 infected ewes at the eleven time points considered in this study and previously cited (from −24 h to 144 h). The blood samples were collected using S-Monovette® 7.5 ml K3E tubes and centrifuged at 2500 ×*g* for 12 min at 4 °C so the plasma could be collected with a pipette, transferred to 1.5 ml microtubes and stored at −80 °C. Later, the plasma samples for all the animals and time points considered in the study were sent in dry ice to the Interactions Hôtes-Agents Pathogènes (**IHAP**) research unit, where they were analysed with a commercially available multiplex immunoassay (MILLIPLEX® MAP Ovine Cytokine/Chemokine Panel 1, EMD Millipore Corp., Billerica, MA, USA) according to the manufacturer's instructions. This is an ovine cytokine/chemokine bead-based multiplex cytokine panel, which, based on Luminex xMAP technology, utilises antibodies against bovine IFN-γ, interleukin (**IL**)-1α, IL-1β, IL-4, IL-6, IL-10, IL-17A, macrophage inflammatory protein (**MIP**)-1α or CCL3, MIP-1β or CCL4, IL-36 receptor antagonist (**IL-36RA**), CXCL10, tumour necrosis factor (**TNF**)-α, and vascular endothelial growth factor (**VEGF**)-A and ovine CXCL8 to screen stored ovine plasma. Measurements of the 14 investigated cytokine/chemokine biomarkers were obtained as pg/mL.
3. Measurement of SCC: Individual milk samples from both mammary glands (approximately 50 mL) were collected from each animal at seven time points around the LPS injection (−24 h, 0 h, 6 h, 24 h, 48 h, 72 h, and 144 h). Milk samples were kept refrigerated during transportation from the farm to the reference laboratory for milk analysis (CENSYRA of León, Spain), where they were analysed for SCC within 24 h after sampling. As previously stated, the SCC measurement of time point −24 h was considered to select the half udder to be injected with LPS, whereas the other gland was considered contralateral.

2.1.3. Data processing and statistical analysis

First, to evaluate potential significant differences in the MY_{adj90d}, PP_{adj90d} and FP_{adj90d} estimations between the animals subjected to the NPR ($n = 20$) and those in the control group (n = 20), a multivariate general linear model (**GLM**) was performed using SPSS software (IBM Corp, 2019). The model included the nutritional challenge (NPR vs. C) group as a fixed effect.

Second, for the data collected for local and systemic indicator traits of inflammation in the 24 animals subjected to the experimental inflammatory challenge (13 NPR vs. 11C) and after assessing the normality of the distributions using the Shapiro–Wilk test (IBM Corp, 2019) (data not shown), nonparametric methods were considered for further analyses. For the SCC trait, we considered a logarithmic transformation (**logSCC**).

To assess the evolution of the measured traits across the 11 considered sampling time points, the raw value of each measurement (whose mean values and standard deviation for each of the considered sampling time points are shown in Supplementary Table S3) was transformed into a ratio trait referencing the basal reference measure. For each phenotype and time pair combination, the basal reference value was defined as the average between the −24 h and 0 h trait values, as indicated in Formula (1).

$$Ratio\ Trait\ 1 = \frac{raw\ data\ Trait1\ (0h/2h/4h/\ldots 144h)}{(raw\ data\ Trait1\ (-24h) + raw\ data\ Trait1\ (0h))/2} \quad (1)$$

By applying this transformation, we defined the ratio traits for the seven sampling points of the two phenotypes related to the local inflammatory response of the mammary gland, the logSCC in the contralateral udder (**r_logSCC_c**) and the logSCC in the LPS-treated udder (**r_logSCC_i**). The ratio transformation was also applied to the 11 measurements collected for each systemic phenotype, the rectal T^{ra}, and the 14 plasma biomarker concentration values.

To analyse the relative importance of the different studied traits throughout the inflammatory challenge, we performed 17 generalised linear model (**GzLM**) analyses using SPSS software (IBM Corp, 2019). The model included the fixed effects on the 11 considered sample collection times (−24 h, 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 72 h, and 144 h) and the challenge (NPR vs. C) group. For the statistical significance level to be considered in the GzLM analysis, a Bonferroni correction considering the number of independent traits analysed as determined by a principal component analysis was performed. Hence, for a total of ten independent traits that were found to explain 90% of the total phenotypic variance, the considered significance threshold was defined as $P \leq 0.005$. An additional analysis was performed for the plasma biomarker concentration records using sparse partial least squares-discriminant analysis (**sPLS-DA**) (Lê Cao et al., 2011). In this analysis, the independent variables (X) considered were the ratio traits of the 14 biomarkers analysed at each time point (154 biomarker measurements), and the dependent variable (Y) was a single qualitative variable representing the NPR and C groups. The sPLS-DA identified a

small subset of variables that best discriminates the two groups. A threefold repeated 50 times cross-validation procedure was performed to determine the number of components to retain and the optimal number of explanatory variables. The "mixOmics" package (Rohart et al., 2017) of R software was used to represent the dispersion and discrimination of the samples and plot the results.

3. Results

3.1. Effects of the nutritional challenge on milk production traits

Considering the data for the milk production traits adjusted to 90 days (mean and standard deviation (SD) for the C and NPR groups are given in Supplementary Table S2), the multivariate GLM analysis for milk production traits did not show significant differences either for the estimated MY_{adj90d} ($P = 0.490$) or for milk composition traits ($P = 0.264$ and 0.482 for PP_{adj90d} and $FP_{adj90d,}$ respectively) between the two studied groups (NPR vs. C).

3.2. Evolution of local and systemic inflammatory traits

In relation to the inflammatory challenge, visual inspection of the udders of the inoculated animals revealed, after LPS injection, mild signs of local inflammation, such as redness and pain, on palpation in the LPS-treated half-udder, whereas no signs of local inflammation were observed in the contralateral half-udder. A detailed description of the evolution of the studied local and systemic markers across the 11 time points considered in this study is given in Supplementary Material S2. A graphical representation of this evolution for the local logSCC indicator trait and the systemic traits analysed, considering all 24 animals, is given in Supplementary Material S1 (Fig. S2 (A-B). The profiles of the ratio traits defined based on the reference basal values, and distinguishing the two groups of the nutritional challenge experiment, are shown in Fig. 1, for the local indicator traits (r_logSCC_i and r_logSCC_c), and in Fig. 2, for the systemic traits (r_T^a and r_cytokine/chemokine traits).

The GzLM analysis performed to assess whether the nutritional challenge influenced local or systemic markers of inflammation also provided a statistical assessment of the relevance of the dynamic changes of the traits across the sampling points analysed (Table 2). Briefly, the dynamics of the indicator traits of local inflammation, time points 6 h, 24 h, 48 h, and 72 h were highly significant ($P < 0.005$) for the r_logSCC_i trait. In contrast, time points 0 h, 6 h, 24 h, and 144 h were also highly significant for the r_logSCC_c ratio trait (Table 2). This finding agrees with the peak values of the raw data of the logSCC trait shown in Supplementary Material S1 (Fig. S2.A) for both the contralateral and the LPS-treated udder. For the systemic traits considered, the most significant time points for the r_T^a trait were 6 h and 8 h ($P < 0.000$). Regarding the plasma biomarkers, the most significant times were between 6 h and 8 h for r_IL-6 ($P < 0.000$) and between 4 h and 6 h for r_IL-10 ($P < 0.000$). Similarly, r_VEGF-A showed significant time points at 4 h and 6 h ($P = 0.002$). Our results suggested that the r_IL-36RA ratio trait is an early response marker with significant effects at 2 h and 4 h ($P = 0.001$ at both time points), whereas r_CXCL8 would be the most significant marker of late response with the most significant effects at 24 h ($P = 0.001$) (Table 2). The significant time points for the systematic biomarkers anlayzed are highlighted with black circles on the x-axis in the plots included in Fig. 2.

3.3. Effects of nutritional challenge on inflammatory traits

In relation to the effect of the NPR/C group on the different inflammatory markers considered here, the GzLM did not identify a significant effect on the considered local indicator traits, the r_logSCC_i or r_logSCC_c traits (Table 2). For the r_T^a trait, the NPR effect was not significant, whereas 8 out of 14 plasma markers were significantly affected by the NPR group ($P < 0.005$; Table 2): r_IFN-γ, r_IL-1α, r_IL-1β, r_IL-4, r_IL-10, r_IL-17A, r_TNF-α, r_VEGF-A. For all these significant results, the influence of the diet restriction effect showed the same direction (negative likelihood estimated for the NPR group) indicating that the C group had higher relative concentration values than the NPR group for the corresponding relative ratio traits (see plots in Fig. 2, where the plasma biomarkers with a significant influence of the nutritional challenge group are highlighted with a black star). In addition, the sPLS-DA analysis applied to the dataset of the 14 plasma biomarker ratio traits at each sampling time point and in relation to the two groups of nutritional challenge (C and NPR) selected one component for the sPLS-DA discriminating groups, whereas the optimal number of selected variables for component 1 was 20. The individual plot of PC1 and PC2, which is given in Fig. 3, shows the separation of the sample groups. As it can be seen, PC1 (20% of total variability) allows the discrimination of one group from the other, whereas PC2 (7% of total variability)

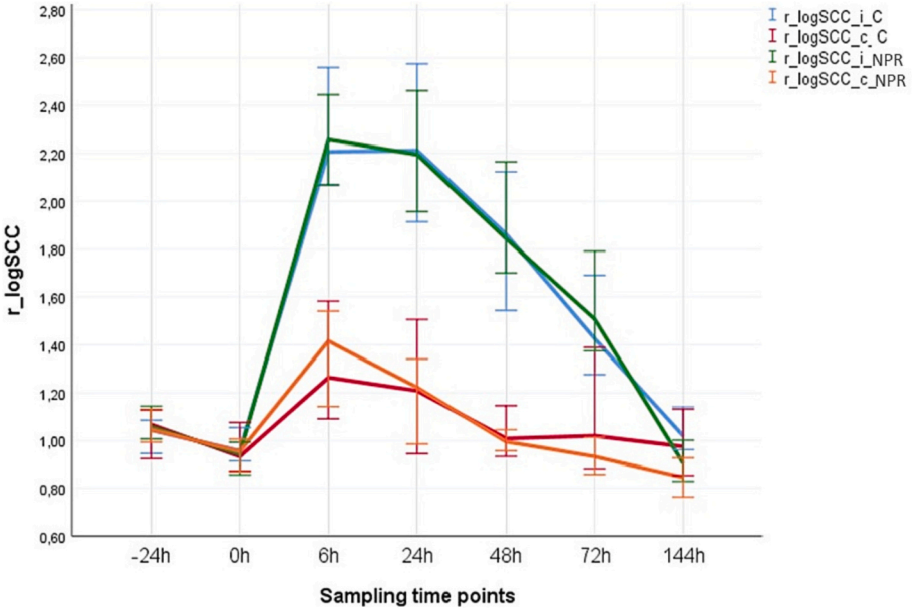

Fig. 1. Graphical representation of the profile of logarithm of the somatic cell count ratio (r_logSCC) measured in both the LPS-injected udder (r_logSCC_i) and the contralateral udder (r_logSCC_c) across the seven milk sampling points considered in the present study for the two studied groups, the nutritional protein restriction (NPR) group (NPR; $n = 13$) and the control group (C; $n = 11$). Note: The ratio traits were calculated considering the measurement corresponding to each specific time point regarding the average between the −24 h and 0 h measurements, which was considered the basal time point. Error bars show the 95% confidence interval of the median.

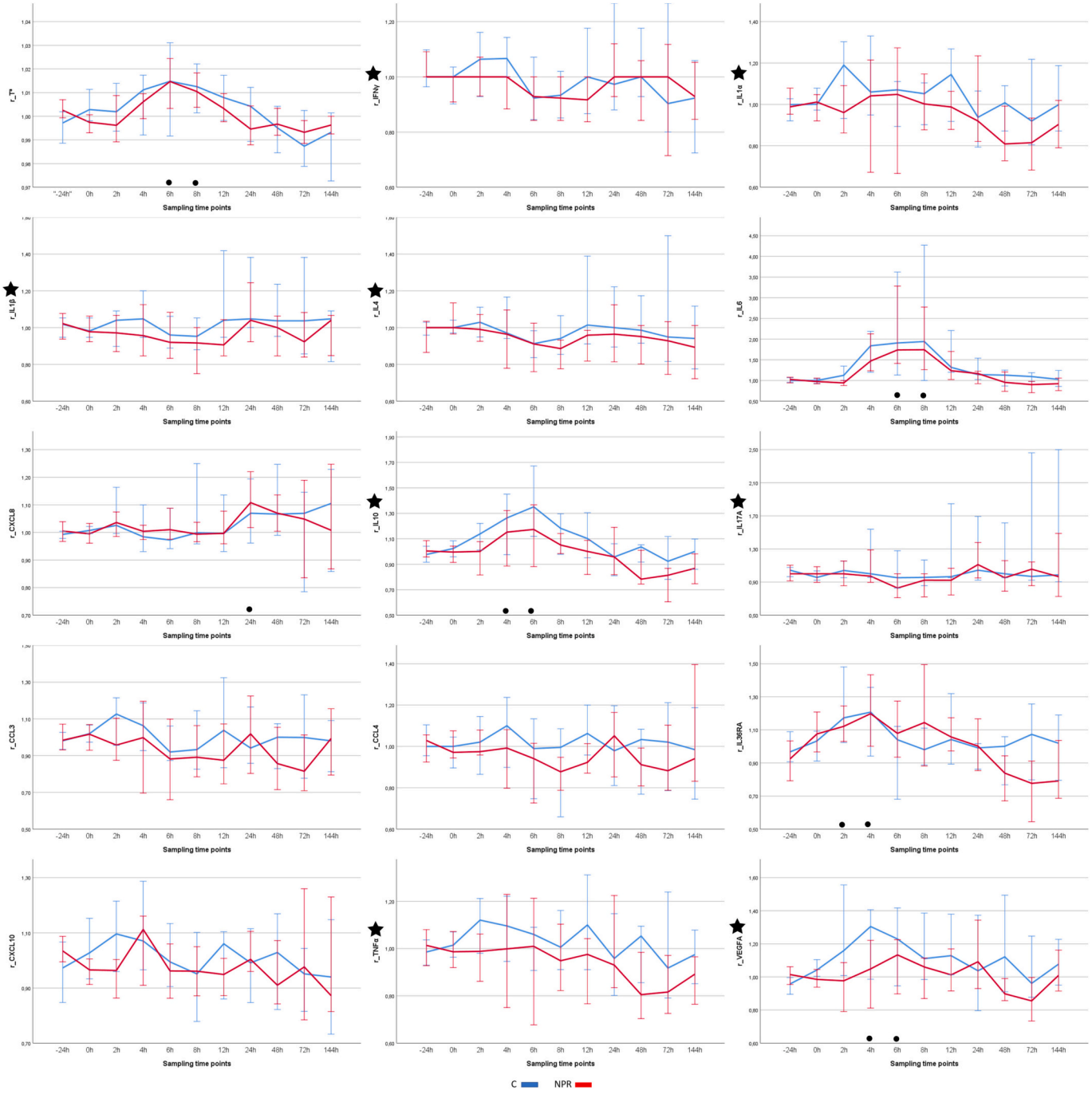

Fig. 2. Graphical representation of the temperature and the 14 cytokine/chemokine ratio traits across the eleven time points considered in the intramammary LPS injection experiment reported here and according to the distribution of the animals in the two groups, the nutritional protein restriction group (NPR; n = 13) and the control group (C; n = 11), represented in red and blue, respectively. Error bars show the 95% confidence interval of the median. The significant results of the GzLM analyses performed are indicated in the figure with a black circle (significant sample collection time points) and a black star (significant effect of the nutritional challenge group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

illustrates the dispersion of the NPR group relative to the C group. The estimates of the variable importance in the projection (**VIP**) for the tested variables analysed in the sPLS-DA are displayed in descending order in Supplementary Table S4. Among the 154 variables tested (i.e., the 14 ratios of the cytokines for each of the 11 time points), 20 were the most discriminant variables between the NPR and C groups for discrimination (VIP > 1.45). The variable with the highest VIP value was r_VEGFA_2 h (2.17). Regarding the time points associated with these discriminant variables, 2 h was associated with seven of the 20 variables with VIP > 1.45.

4. Discussion

The increased cost of animal dietary protein and the legislation related to the storage and application of manure from different species has promoted interest in reducing protein levels in diets (Sinclair et al., 2014). Few studies on dairy ewes have considered the impact of dietary protein restriction. Therefore, the present work was conceived to determine whether a diet restriction performed in ewe lambs, coinciding with the allometric growth of the mammary glands, could influence future milk production or even the susceptibility to mastitis later in their

Table 2
Effects of changes in inflammatory indicators according to different time points and control (C) or nutritional challenge (NPR) groups based on the results of the generalised linear model (GzLM) analyses. Estimates are given for the basal level (intercepts) and predictors. The maximum likelihood estimate (B) and the error (se) are shown for each trait.

Traits		-24 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h	48 h	72 h	144 h	C	NPR
r_logSCC_i	B	0[1]	−0.09			1.22 [a]			1.16 [a]	0.80 [a]	0.43 [a]	−0.07	0[1]	−0.00
	se		0.06			0.06			0.06	0.06	0.06	0.06		0.03
r_logSCC_c	B	0[1]	−0.13[a]			0.27 [a]			0.16 [a]	−0.05	−0.07	−0.12 [a]	0[1]	−0.06
	se		0.04			0.04			0.04	0.04	0.04	0.04		0.02
r_T[a]	B	0[1]	0.00	0.00	0.01	0.01 [a]	0.01 [a]	0.01	−0.00	−0.00	−0.01	−0.01	0[1]	0.00
	se		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
r_IFN-γ	B	0[1]	−0.11	0.02	−0.02	−0.16	−0.13	−0.04	−0.06	−0.09	0.01	−0.12	0[1]	−0.10 [a]
	se		0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08		0.03
r_IL-1α	B	0[1]	0.03	0.05	0.06	0.04	0.02	0.06	−0.02	−0.09	−0.12	−0.04	0[1]	−0.09 [a]
	se		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05		0.02
r_IL-1β	B	0[1]	−0.04	−0.02	0.02	−0.06	−0.07	0.01	0.05	−0.03	−0.02	0.02	0[1]	−0.08 [a]
	se		0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06		0.02
r_IL-4	B	0[1]	0.01	0.03	0.00	−0.08	−0.08	−0.00	−0.01	−0.05	−0.01	−0.06	0[1]	−0.07 [a]
	se		0.05	0. 05	0. 05	0. 05	0. 05	0. 05	0. 05	0. 05	0. 05	0. 05		0.03
r_IL-6	B	0[1]	−0.04	0.01	0.76	1.51 [a]	1.59 [a]	0.39	0.29	0.02	−0.04	−0.07	0[1]	−0.02
	se		0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28		0.12
r_CXCL8	B	0[1]	0.01	0.06	0.03	0.02	0.02	0.04	0.11 [a]	0.09	0.03	0.06	0[1]	0.00
	se		0.03	0.03	0.03	0.03	0.06	0.03	0.03	0.03	0.03	0.03		0.01
r_IL-10	B	0[1]	0.02	0.04	0.22 [a]	0.32 [a]	0.11	0.04	−0.03	−0.10	−0.14	−0.06	0[1]	−0.11 [a]
	se		0.06	0.06	0.06	0.06	0.03	0.06	0.06	0.06	0.06	0.06		0.03
r_IL-17A	B	0[1]	−0.06	0.02	0.06	−0.11	−0.08	0.08	0.17	0.01	0.09	0.19	0[1]	−0.15 [a]
	se		0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11		0.05
r_CCL3	B	0[1]	−0.02	0.02	0.03	−0.05	−0.08	0.00	−0.01	−0.10	−0.11	−0.04	0[1]	−0.08
	se		0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06		0.03
r_CCL4	B	0[1]	−0.05	0.01	0.02	−0.11	−0.11	−0.03	−0.01	−0.09	−0.06	−0.02	0[1]	−0.06
	se		0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06		0.02
r_IL-36RA	B	0[1]	0.08	0.20 [a]	0.20 [a]	0.07	0.11	0.12	0.07	−0.07	−0.07	−0.05	0[1]	−0.03
	se		0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06		0.03
r_CXCL10	B	0[1]	0.02	0.02	0.08	−0.01	−0.03	−0.02	0.03	−0.02	0.00	−0.01	0[1]	−0.01
	se		0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04		0.02
r_TNF-α	B	0[1]	0.02	0.02	0.05	0.01	−0.02	0.03	−0.02	−0.11	−0.13	−0.07	0[1]	−0,09[a]
	se		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05		0.02
r_VEGF-A	B	0[1]	0.03	0.09	0.18 [a]	0.18 [a]	0.09	0.12	0.11	0.06	−0.06	0.06	0[1]	−0.12 [a]
	se		0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06		0.02

[1] Set to zero because this parameter is redundant.
[a] Significant result considering the Bonferroni correction applied (*P* value <0.005).

productive life. The nutritional challenge was planned to mimic the conditions of a commercial flock. Hence, the only difference between the diets given to the C and the NPR groups was the presence or absence of soybean meal protein supplementation.

Castro-Costa et al. (2014) also performed an intramammary *E. coli* LPS infusion in Lacaune dairy ewes at late lactation and reported results in agreement with those described here for both local and systemic inflammation indicators. The discussion presented below, which is focused on understanding the changes in the induced inflammatory response across different time points, is necessary for a proper interpretation of the effects of dietary restriction on those parameters, as presented later.

4.1. Evolution of inflammatory markers across time sampling points

The local inflammatory response reported in this work after intramammary LPS infusion agrees with that reported in cows by Rainard et al. (2013), who found that the infusion of microbial-associated molecular patterns, such as LPS, into the cistern of the mammary gland induced an inflammatory response lasting approximately 72 h. Our results also agree with those previously described in the intramammary inflammation model reported in sheep by Castro-Costa et al. (2014), which, in addition to significant changes in the concentration of milk components 6 h after the LPS challenge, found a dramatic increase in the milk logSCC that remained high until 72 h after the LPS challenge. It is well known that the primary defence mechanism against infection at the early stage is leucocyte recruitment to the affected area, which in the case of the mammary gland produces an exponential increase in milk SCC due to leukocyte diapedesis to the mammary tissue (Bochniarz et al., 2017).

In addition to the local response, in mammals, the acute-phase reaction is a prominent nonspecific reaction of the organism at the systemic level to local or systemic disturbances that manifests as a rapid increase in the production of proteins, recognised as markers of inflammation (Murata et al., 2004). By assessing the kinetics of systemic inflammatory markers (rectal temperature and blood concentrations of cytokines/chemokines), we showed that the dose of intramammary LPS inoculated here also elicited a systemic response. Specifically, the LPS infusion triggered an acute body temperature response, with the maximum at 6 h post-inoculation. This agrees with results reported in female lambs subjected to an intravenous bolus of LPS in a systemic challenge (Naylor et al., 2020). Likewise, the intramammary LPS challenge reported in Lacaune dairy ewes highlighted the 6 h post-LPS-inoculation time point as the highest peak for the temperature records obtained for both vaginal and udder skin (Castro-Costa et al., 2014).

Regarding the analysed plasma biomarkers, the r_IL6 ratio trait showed the highest increase relative to the considered basal trait value, with the highest peak and the most significant results detected between 6 h and 8 h post-LPS injection. The increase in body temperature or fever is a stereotypical response to infection and is thought to be driven by some proinflammatory cytokines, particularly IL-1, IL-6, and TNF-α (Naylor et al., 2020). Specifically, IL-6 was identified in in vitro and in vivo studies as the primary inducer of hepatic synthesis of acute-phase proteins with proinflammatory properties (Bochniarz et al., 2017). It is worth mentioning that r_IL-6 showed a similar profile to the r_SCC_trait; therefore, plasma IL-6 concentration could be considered an early systemic biomarker of acute mastitis in sheep. This finding agrees with previous studies on dairy cows, suggesting that IL-6 concentrations

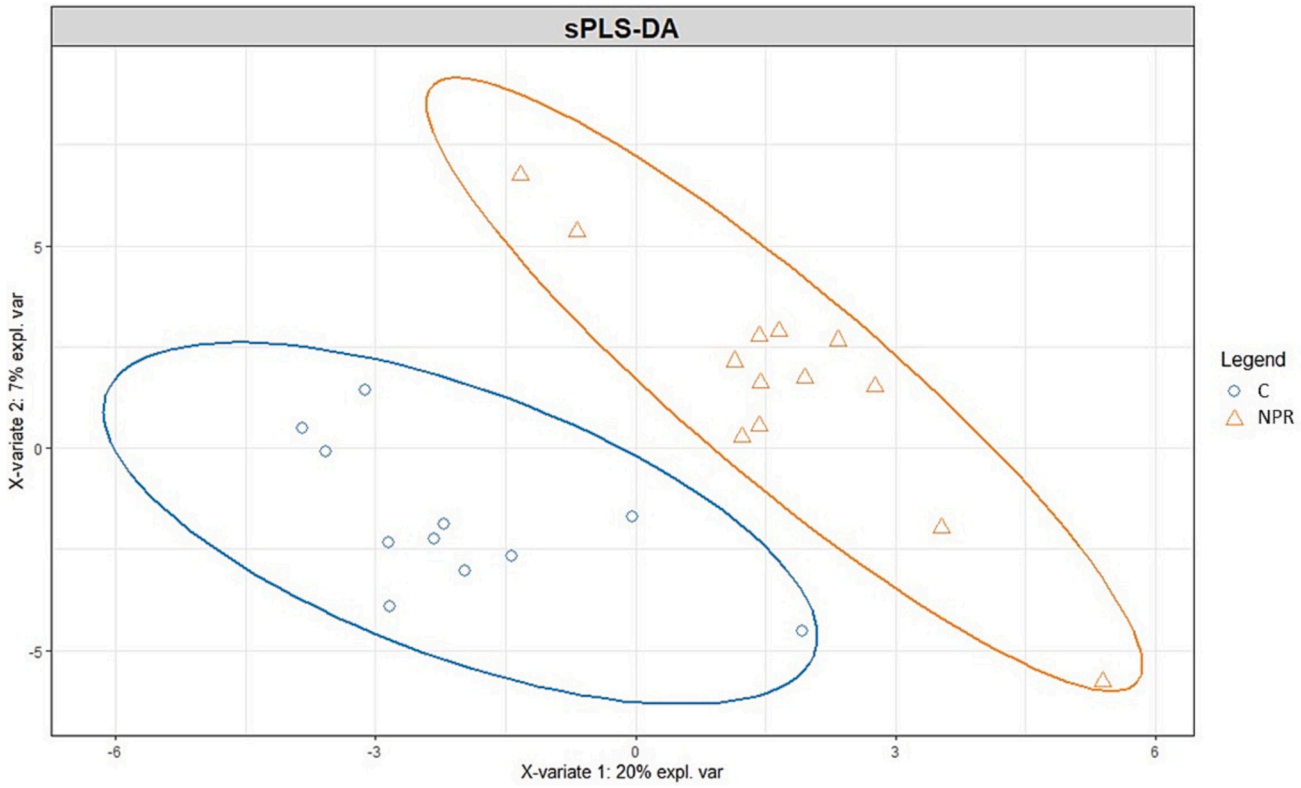

Fig. 3. Individual plot of sparse partial least squares discriminant analysis (sPLS-DA) for the studied data with the first two components. The different colours indicate the different groups: blue for the control group (C; n = 11) and orange for the nutritional challenge group (NPR, n = 13). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in milk (Sakemi et al., 2011) and serum (Bochniarz et al., 2017) indicate subclinical mastitis. The lack of significant results for other proinflammatory cytokines analysed in this work (e.g., IL-1 α, IL-1β, IL17A, and TNF-α) may be explained by the low LPS dose applied here, similar to the results reported by Naylor et al. (2020).

Among the anti-inflammatory cytokines included in the analysed multiplex immunoassay, the observed increased concentration of IL-10, an important immunoregulatory cytokine, is consistent with the results reported in LPS-induced endotoxaemia, where the IL-10 serum levels reached their maximum at approximately 4 h after LPS challenge (Naylor et al., 2020). In both studies, the experimental challenge used LPS from a gram-negative bacterium. The early peak observed for this cytokine agrees with the observations related to intramammary infections in dairy cows showing earlier increases in IL-10 production in response to gram-negative bacteria than to gram-positive bacteria (Bannerman, 2009). Additionally, with anti-inflammatory effects, IL-36RA and VEGF-A showed oscillating concentrations throughout the experiment, with two significant sampling points being identified for each of them (2 h and 4 h for IL-36RA and 4 h and 6 h for VEGF-A; Table 2). IL-36RA inhibits and limits the proinflammatory effects of interleukin-36 family-related cytokines (i.e., IL-36α, IL-36β, and IL-36γ) (Towne et al., 2004). In contrast, VEGF-A is a master regulator of vascular homeostasis and angiogenesis whose main function is to promote vasodilation, angiogenesis, vascular permeability, and homeostasis (Pandey et al., 2018).

In addition, chemokines are a large family of small secreted proteins that stimulate cell migration, most notably white blood cells (Hughes and Nibbs, 2018). Most of the chemokines analysed in the present work (r_IFN-γ, r_CCL3, r_CCL4, and r_CXCL10) showed no observable responses over time. Nevertheless, neutrophil infiltration into inflammatory sites is one of the hallmarks of acute inflammation, and the chemokine CXCL8 has been shown to recruit neutrophils selectively during *E. coli*-induced mastitis in sheep (Gangur et al., 2002). Our results showed a significant increase in the concentration of this chemokine (r_CXCL8) at 24 h after LPS injection (see Table 2 and Fig. 2), consistent with the review of Kaplanski et al. (2003), who reported that when inflammatory cytokines stimulate neutrophils or other cells, CXCL8 is produced over 24 h, locally recruiting and activating neutrophils. Hence, CXCL8 could be a later biomarker of the inflammatory response in the ovine mammary gland.

4.2. *Effects of prepuberal protein restriction on milk production and inflammatory-related traits at first lactation*

Focusing now on the effects of the nutritional challenge performed in the prepuberal ewes studied here, and despite the limited number of animals analysed in the present study, our results suggest that the dietary protein restriction of the studied ewe lambs did not affect their first lactation milk production and milk composition traits, as no significant differences were found for these traits between the two compared groups (NPR vs. C). These results agree with those reported by Zhang et al. (1995), who did not find significant differences in the first lactation milk yield between prepuberal ewes fed 15% vs. 20% protein. Although these authors found that ewes fed a 20% protein diet showed an enhanced mammary gland weight and a numerically nonsignificantly greater milk yield, they suggested that these observations were more likely the result of changes in cell numbers than in secretory cell activity. Hence, it seems that the effect of a protein-restricted diet in prepubertal ewes performed in a commercial flock would not have a negative impact on the animal's milk yield potential at the first lactation, although further studies would be needed to confirm this statement.

Regarding the results of the GzLM analysis concerning the NPR effect on the local inflammatory response, our results suggest that protein restriction at prepubertal age does not significantly influence the local inflammatory response induced by LPS, measured through the SCC indicator trait (Table 2). In dairy species, we have not found studies

assessing the effects of nutritional restriction at prepubertal age on the response to udder inflammation later in life. Nevertheless, our results appear to be in line with those reported by cattle studies assessing the effect of pre- and postpartum protein nutrition that found no major effects on SCC, the incidence of mastitis or other diseases (Sinclair et al., 2014).

In terms of the systemic inflammatory response triggered by intramammary LPS inoculation, the NPR group effect did not affect the rectal temperature, whereas it was significant for eight of the 14 plasma biomarkers analysed (Table 2). Interestingly, as previously said, the relative concentrations were higher in the C group for all eight biomarkers significantly influenced by the dietary restriction. Looking at the individual profiles of these significant biomarkers (marked in Fig. 2 with a black star), we can see that the higher concentration of some of these indicators in the C group, compared with the NPR group, was already observed at early time points (e.g., r_ IFN-γ, r_ IL-1α, r_ IL-1β, at 2–4 h). This observation is reinforced by the discriminant analysis results, where the 2 h sampling point was highlighted as the most important point to discriminate between both groups.

From these eight plasma biomarkers showing significant differences between the two groups, only two of them, VEGF-A and IL-10, had shown significant changes (at 4 h and 6 h) in our previous assessment of the dynamic evaluation of the inflammatory response. Looking for potential links between these two regulators of the inflammatory response and the prepubertal nutritional challenge studied here, we would like to highlight that VEGF-A has been reported to play a critical role in the mammary gland development and function during pregnancy and lactation by Rossiter et al. (2007). These authors showed that inactivation of VEGF-A in the mammary gland epithelium during pregnancy resulted in a reduced blood vessel density and, perhaps more importantly, in a functional inadequacy of the blood vessels, which led to ineffective delivery of fluid, hormones, and other substances. In addition, different studies in rats have shown that maternal protein restriction alters VEGF-A signalling, which modifies the development of different organs in the fetus (Liu et al., 2014; Cavariani et al., 2019). Additionally, dietary protein sources have been related to tumoral overexpression of VEGF-A in breast cancer patients (Shokri et al., 2019). All this would support the hypothesis that the dietary protein restriction performed in our study may have altered the development of the mammary gland of the NPR ewes in relation to the role of the VEGF-A signalling pathway on the angiogenesis and blood vessel density. The crucial role that VEGF-A plays in regulating the permeability of mammary gland blood vessels would explain that the animals in the C group showed higher concentrations of this plasma biomarker and all the other seven cytokines/chemokines affected by the nutritional challenge. This would also be supported by the fact that in the discriminant analysis, the variable showing the highest VIP value was VEGF-A_2 h.

Concerning IL-10, different studies in humans have shown a relationship between circulating IL-10 levels and metabolic traits (Esposito et al., 2003; Hong et al., 2009). IL-10 is produced by a wide-range of immune cells (e.g., macrophages, dendritic cells, T cells, B cells, mast cells, and neutrophils) in response to inflammatory signals, and classically has been reported to elicit a systemic anti-inflammatory response (Hutchins et al., 2015). However, there is recent evidence that IL-10 may play a dual role, in some contexts stimulating the immune response instead of suppressing it, depending on specific cell types and contexts (Saraiva et al., 2019). All this suggest that IL-10 is a regulatory cytokine of the immune response. Resolution of infection requires a coordinated response where initial pro-inflammatory mechanisms clear the pathogen and are then down-modulated by IL-10 before pathology occurs. Thus, the relative amounts of pro-inflammatory and anti-inflammatory cytokine production are critical for a safe resolution of infection (Couper et al., 2021). Based on this regulatory action of IL-10, we have considered of interest to assess if the two studied groups would show any difference on the functional activity of the mammary gland at the time of the inflammatory challenge. For that, we performed a GLM analysis for five available measurements of milk yield recorded at different points before (−12*h*,0*h*) and after (6 h, 24 h, and 48 h) the LPS inoculation, including the nutritional challenge (NPR vs. C) group as a fixed effect. The results of this analysis (provided as Supplementary Table S5) showed that, whereas a clear reduction of the milk production was observed in the two groups at 6 h after the LPS inoculation, a significant difference between groups was detected only at the 24 h time point, with the C group showing a higher average milk production. Based on the average milk yield measurements, the functional activity of the mammary gland was recovered at 48 h.

Overall, the results described here in relation to the inflammatory challenge would suggest that, after the LPS inoculation, the animals in the C group, although they did not show significant differences in the SCC local indicator trait, have shown a faster inflammatory response at the systemic level than the animals of the NPR group. As commented before, our observations point to the VEGF-A biomarker as a potential cause mediating these results. This differentiated systemic response appear to be also associated with a minor alteration of the functional activity of the mammary gland in the C group, which could be explained by higher systemic levels of the cytokine IL-10 when compared with the NPR animals. In any case, we acknowledge that the results of this work, based on a limited number of animals, would need to be confirmed by future studies.

5. Conclusion

The results reported here suggest that the protein restriction performed in prepuberal ewe lambs does not affect the animals' milk production potential later in life, and that when subjected to an inflammatory LPS challenge of the mammary gland, the protein-restricted diet has not altered the local inflammatory response, measured through the SCC indicator trait. However, after the LPS challenge, significant differences were found for eight plasma biomarkers and for the average milk yield at 24 h post LPS inoculation between the two studied groups (C and NPR). Based on the dynamic evolution of the inflammatory response described here, two of the significantly affected plasma biomarkers, VEGF-A and IL-10, have been highlighted as potential regulators to explain the differences observed between the two groups. Specifically, the critical role of VEGF-A on angiogenesis of tissues under development could suggest that the dietary protein restriction here studied might have had some effects at the time of the development of the mammary gland in the prepubertal NPR ewes, and this could influence later in life the systemic inflammatory response triggered under an inflammatory challenge of the mammary gland. Likewise, the regulatory action of the IL-10 cytokine could be related with the faster recovery of the functional ability of the mammary gland after the LPS challenge. Further complementary studies should be undertaken to confirm these results and fully understand the consequences of a dietary protein restriction in replacement ewes.

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Declaration of Competing Interest

None.

Data availability

All data are available on request to the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.04.006.

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